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GENOME RESEARCH

July 1996

Volume 6 Number 7

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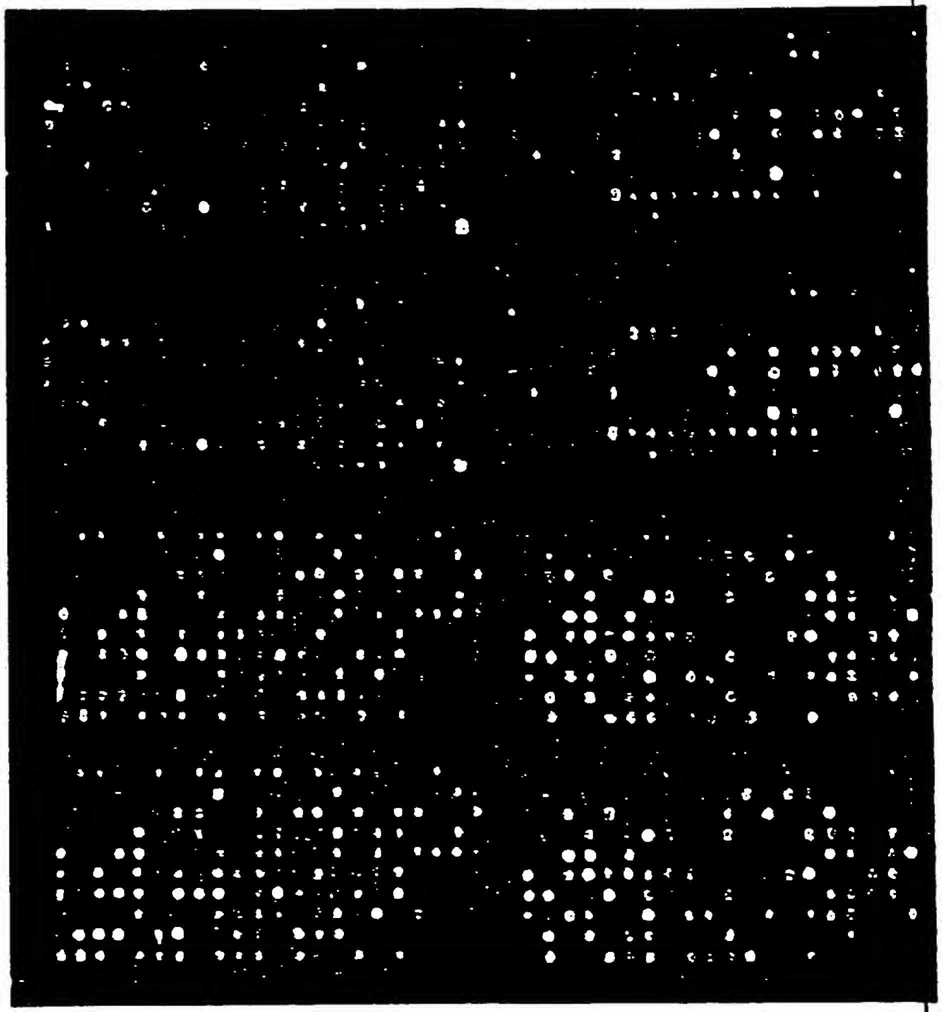
Human Y Chromosome Haplotypes

BAC Mapping of Extrachromosomal Structure

DNA Microarray System



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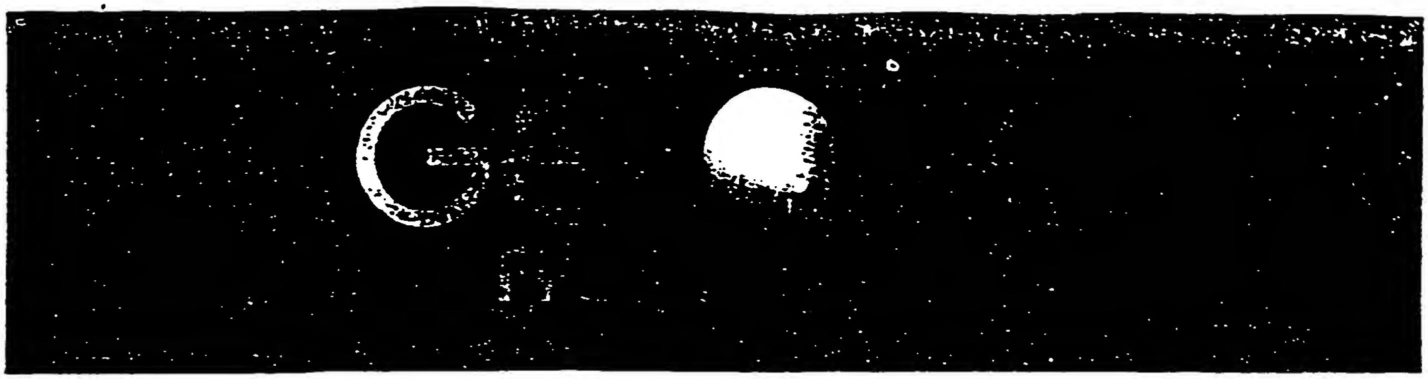

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GENOME RESEARCH (ISSN 1054-9803) is published monthly for $495 (U.S. institutional; $545 R.O.W.), $95 (individual making personal payment; $145 R.O.W., includes airlift) by Cold Spring Harbor Laboratory Press, 1 Bungtown Road, Cold Spring Harbor, New York 11724. Periodicals class postage pending is paid at Cold Spring Harbor and additional mailing offices. POSTMASTER: Send address changes to Cold Spring Harbor Laboratory, 10 Skyline Drive, Plainview, New York 11803-2500.

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GENOME RESEARCH

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A DNA Microarray System for Analyzing Complex DNA Samples Using Two-color Fluorescent Probe Hybridization

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Detecting and determining the relative abundance of diverse individual sequences in complex DNA samples is a recurring experimental challenge in analyzing genomes. We describe a general experimental approach to this problem, using microscopic arrays of DNA fragments on glass substrates for differential hybridization analysis of fluorescently labeled DNA samples. To test the system, 864 physically mapped λ clones of yeast genomic DNA, together representing >75% of the yeast genome, were arranged into 1.8-cm × 1.8-cm arrays, each containing a total of 1744 elements. The microarrays were characterized by simultaneous hybridization of two different sets of isolated yeast chromosomes labeled with two different fluorophores. A laser fluorescent scanner was used to detect the hybridization signals from the two fluorophores. The results demonstrate the utility of DNA microarrays in the analysis of complex DNA samples. This system should find numerous applications in genome-wide genetic mapping, physical mapping, and gene expression studies.

Many problems in genome analysis depend on determining what specific sequences are represented in a complex DNA or RNA sample and at what abundance, for example, what genes are represented in a specific chromosome band or YAC clone, what intervals are amplified or deleted in a particular cancer cell, or what genes are expressed in specific cells under specific conditions. As a general approach to this problem, we have developed a system for making microarrays of DNA samples on glass substrates, probing them by hybridization with complex fluorescent-labeled probes, and using a laser-scanning microscope to detect the fluorescent signals representing hybridization. Fluorescent labeling allows for simultaneous hybridization and separate detection of the hybridization signal from two or more probes. This in turn allows very accurate and reliable measurement of the relative abundance of specific sequences in two complex samples.

RESULTS

Array Hybridization Pattern

Figure 1 shows the two-color fluorescent scan of a yeast genomic array following hybridization with a mixed probe consisting of lissamine-labeled DNA from the 6 largest yeast chromosomes together with fluorescein-labeled DNA from the 10 smallest yeast chromosomes. A red color indicates that yeast sequences present in the lissamine-labeled hybridization probe hybridized to an array element. A yellow-green color indicates that yeast sequences present in the fluorescein-labeled hybridization probe hybridized to an array element. An orange color indicates cross-hybridization of both chromosome pools to an array element (e.g., dispersed repetitive elements, such as Ty1 elements).

Each clone was spotted twice, resulting in duplicate hybridization patterns in adjacent quadrants of the array. Control DNA spots, which were randomly amplified in the same manner as the λ clone array elements, are located in the bottom corner of each quadrant. "A" points to a pair of spots containing total yeast genomic DNA. These spots appear orange because both chromosome pools hybridized to yeast genomic DNA. The negative controls are as follows: "B" points to a pair of spots of wild-type λ DNA, "C" points to a pair of human genomic DNA spots, and "D" points to a pair of ϕX174 DNA spots. The lack of a hybridization signal at these three negative control spots indicates that the hybridization was specific for yeast sequences.

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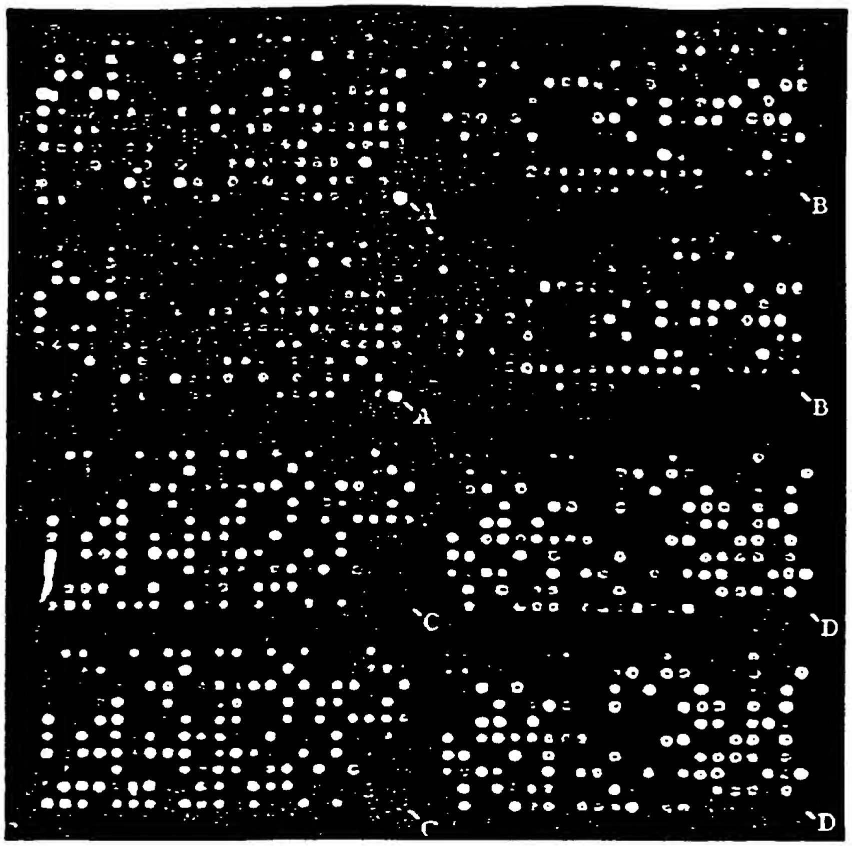

Figure 1 Two-color fluorescent scan of a 1.8-cm × 1.8-cm yeast array of λ clones of yeast genomic DNA. The DNA spots are spaced at a distance of 380 μm from center to center. A probe mixture consisting of DNA from the 6 largest yeast chromosomes (4, 7, 12, 13, 15, 16) labeled with lissamine (red dots) and DNA from the 10 smallest yeast chromosomes (1, 2, 3, 5, 6, 8, 9, 10, 11, 14) labeled with fluorescein (yellow-green dots) was hybridized to the array. A pair of yeast genomic DNA spots (*A*) served as a positive control. The three negative controls are λ DNA (*B*), human genomic DNA (*C*), and ϕX174 DNA (*D*).

Karyotype Depiction of the Array Hybridization Pattern

The inserts contained in the arrayed λ clones have been mapped physically (Riles et al. 1993). The clones are arrayed in a random but known order on the array. Therefore, using the identity of each clone along with its physical map information, the pattern of hybridization to the yeast array can be represented in the form of a karyotype of the yeast genome, as shown in Figure 2. The color of any segment of the ideogram representing an individual chromosome on the karyotype is directly determined by the ratio of red and green hybridization signals at the array positions of the corresponding clones. The lengths of the discrete colored segments of each chromosome correspond to the physical lengths of the yeast inserts. The chromosome segments colored black represent either intervals of the genome that are not represented by clones in the library (90%) or false-negative hybridization signals on the array (10%). Most of these false negatives are attributable to failures of the PCR amplification of the λ clones, though occasional failures of the arraying process or nonuniform surface preparation could account for a small fraction of the false-negative signals. The large gap on chromosome 12 is the region coding for ribosomal DNA that was not represented among the arrayed clones. Genomic intervals represented by overlapping clones were assigned a color based on the hybridization signals of only one of the overlapping clones, chosen at random.

Note that in this representation of a yeast karyotype, the largest six chromosomes are mainly colored red. This indicates that most of the arrayed clones that were mapped previously to these six large chromosomes hybridized primarily to the lissamine-labeled probe prepared from the corresponding purified chromosomes. Conversely, the smallest 10 chromosomes are mainly colored green in this image, matching the original CHEF gel isolation of the chromosomes used as the hybridization probe. The experiment was repeated with the yeast genome split into six discrete chromosome pools containing 2–4 chromosomes per pool using CHEF gel electrophoresis. The chromosomes in each pool were extracted from the gel, amplified, and fluorescently labeled. The six chromosome pools were hybridized to six separate yeast arrays. Forty-four λ clones gave a positive hybridization signal on all six arrays indicating that they contain yeast repetitive sequences (data not shown). These 44 clones and 10 clones with very weak hybridization signals were not included in the data set used to produce this karyotype.

There were ~40 anomalous clones, which appear in this karyotype representation as green bands on the otherwise red chromosomes or red

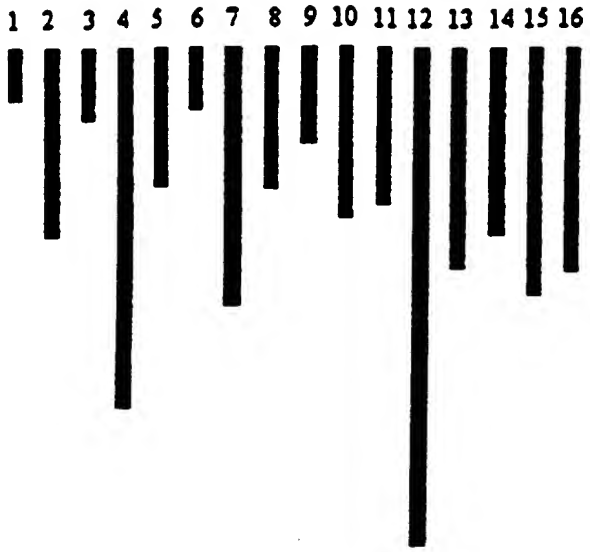

Figure 2 Computer-generated ideogram representing a karyotype of *S. cerevisiae,* based on the normalized hybridization signals from the array shown in Fig. 1. Note that the 6 largest chromosomes are mainly red and the 10 smallest chromosomes are mainly green. Black stripes represent intervals not represented by clones in the array or for which the corresponding clones gave false-negative hybridization signals.

bands on the otherwise green chromosomes. Four randomly chosen examples of these anomalous clones were analyzed by hybridizing the clones to vertical strips cut from a Southern blot of CHEF gel-separated yeast chromosomes. In each case, the hybridization patterns of the anomalous clones corroborated the chromosomal locations assigned by the microarray hybridization results (data not shown). Two clones that were thought to map to the 10 smallest chromosomes were found to hybridize preferentially to the probe representing the 6 largest chromosomes and thus appear as anomalous red bands on the karyotype. Both hybridized to one of the six largest chromosomes on the Southern blot. Similarly, two clones that appear as anomalous green bands on the karyotype were found to hybridize to one of the 10 smallest chromosomes on the Southern blot. Thus, the anomalous clones are probably the result of sample tracking errors or, possibly, of errors in the published restriction-digest-based physical map on which the karyotype representation was based (Riles et al. 1993).

DISCUSSION

The DNA microarray hybridization system reported here is conceptually and functionally similar to fluorescent in situ hybridization (FISH) to metaphase chromosomes, with three important differences. First, the target elements of the microarrays can, in principle, be any length or composition, from megabase YAC clones or microdissected chromosome bands to individual cDNA clones, to short oligonucleotides. This versatility allows the user to choose characteristics, such as the mapping resolution and genetic complexity of each array element, to suit a particular application. Second, the hybridization signals are localized to discrete elements of known size and location, making them easier to identify and quantitate than the hybridization signals from irregularly shaped metaphase spreads. Third, microarrays are more consistent and potentially amenable to automated production, hybridization, and data analysis than metaphase spreads.

Arrays of DNA samples on porous membranes, for example, dot blots, have long been used as a basic tool in molecular biology. Dot-blot membranes are usually at least 8 × 12 cm in size, require the use of milliliter volumes of hybridization solution, and are limited, owing to autofluorescence and scattering, to radioactive, chemiluminescent, and colorimetric hybridization detection methods (Ross et al. 1992). Microarrays made on glass surfaces, on the other hand, can be mass-produced and are comparatively inexpensive, convenient, and compatible with fluorescent hybridization detection methods. Furthermore, a glass surface, when appropriately treated, has very low nonspecific binding of labeled hybridization probes, resulting in lower backgrounds than are encountered typically with porous membranes. For hybridizations with very complex probes, the concentration of the labeled probe DNA is a limiting factor in the sensitivity of the assay. Minimizing the volume of the probe solution in a hybridization, by restricting the target to a small area and by using a nonporous substrate, makes it practical to achieve very high probe concentrations.

One important advantage of fluorescently labeled probes is that, unlike most radioactive and chemiluminescent signals, fluorescent signals do not disperse and therefore allow for very dense array spacing. A unique, and probably the most important, advantage of fluorescent probes is that the hybridization signals from two or more differently labeled probes hybridized to the same target element can be detected separately. In this way, two-color hybridization detection allows for a direct and quantitative comparison of the abundance of specific sequences between two probe mixtures that are hybridized competitively to a single array. The absolute intensity of a hybridization signal at a particular element in an array can vary owing to experimental factors such as variations in the amount of DNA deposited on the array, variations in the hybridization or wash conditions between experiments, or variations in the hybridization characteristics of the different DNA sequences on the array. The ratio of the two signals at any element in an array, however, is relatively insensitive to these confounding factors because they affect both probe mixtures equivalently. This ratio therefore accurately reflects the relative abundance of the cognate sequence in the two probe samples. This is the principle underlying the technique of comparative genomic hybridization (CGH), which is used to detect changes in the copy number of specific chromosomes or chromosomal regions (Kallioniemi et al. 1992). CGH is based on measuring the relative fluorescent hybridization intensities of two genomic-complexity hybridization probes, for example, probes representing genomic DNA from normal and affected tissue samples, which are labeled with two distinct fluorophores and hybridized simultaneously to a metaphase spread. DNA microarray representations of the human genome may provide a more convenient and higher resolution alternative to metaphase chromosomes for CGH.

Cross-hybridization between related sequences is an important problem faced by any hybridization-based assay, including the DNA microarray assay described here. Studies are now in progress to quantitate the extent of cross-hybridization between related sequences of varying homology and length, in DNA microarray hybridizations. The stringency of hybridization and washing can be controlled by varying the salt concentration and temperature as in conventional membrane-based hybridizations. Cross-hybridization caused by repetitive sequences can be minimized by prehybridization of the probe or array with vast excess of unlabeled copies of the repetitive sequences.

Alternative methods have been described for making microarrays of very short DNA sequences, involving photolithography (Pease et al. 1994) or physical masking (Maskos and Southern 1992) methods. These in situ synthesis methods are inherently limited to low complexity array elements consisting of oligonucleotides. F r complex-probe hybridizations, the specificity of hybridization is improved by using DNA fragments substantially longer than oligonucleotides. Moreover, the in situ synthesis approaches to array fabrication depend on prior knowledge of the sequence to be recognized by each array element. The approach described here makes microarrays by transferring tiny volumes of DNA samples from microwell storage plates to a solid substrate. Thus, nucleic acids (or other molecules) of virtually any length or any origin can be arrayed, and knowledge of their sequences is not required.

The arrays used in these experiments do not represent the maximal achievable density of elements. We have found that the spacing between the spots can be decreased by shrinking the contact area of the printing tip and by increasing the hydrophobicity of the glass surface. Microarrays with 100-μm feature size have been tested successfully in pilot experiments (data not shown). Assuming the projected availability of the appropriate physically mapped human genomic clones (Hudson et al. 1995), arrays at 100-μm spacing would allow for 10,000 discrete intervals of the human genome to be represented in a 1-cm^2 array. Such an array could be used for mapping at a resolution of <0.5 Mb. Experiments are in progress to explore the feasibility of such arrays.

Our initial motivation for developing these microarrays arose from the need for abundant and inexpensive genomic arrays for genomic mismatch scanning (GMS) (Nelson et al. 1993), a method of genetic linkage analysis based on identification of the regions of "identity by descent" between affected relative pairs using a single complex-probe hybridization to an array of genomic clones. Experiments using these arrays to map quantitative trait loci in yeast by GMS are currently in progress (J. deRisi, D. Lashkari, L. Penland, L. McAllister, J. McCusker, R. Davis, and P.O. Brown, unpubl.).

Microarrays of cDNA clones, prepared using the system described here, have been used for quantitative monitoring of gene expression patterns in *Arabidopsis* (Schena et al. 1995), *S. cerevisiae* (D. Lashkari, J. deRisi, L. Penland, P.O. Brown, and R. Davis, unpubl.), and human tissues (J. deRisi, M. Bittner, P. Meltzer, L. Penland, J. Trent, and and P.O. Brown, unpubl.). We anticipate that DNA microarrays of the kind described here will be useful in additional applications for which conventional dot blots, high-density gridded arrays on porous membranes, or FISH are currently used. These potential applications include comparative genomic hybridization (Kallioniemi et al. 1992), sequencing by hybridization (Drmanac et al. 1993), physical mapping of cloned or amplified sequences (Billings et al. 1991), and economical distribution of reagents for integrated genetic and physical mapping based on a common set of arrayed clones (Zehetner and Lehrach 1994).

METHODS

Amplification of Target DNA Elements

The array elements were prepared from physically mapped λ clones (Riles et al. 1993). The λ clones were amplified using randomly primed polymerase chain reaction (PCR) based on published and unpublished protocols (Bohlander et al. 1992; S. Nelson, unpubl.). The phage lysates were amplified in a 10-μl PCR reaction using 5 μM final concentration of primer A (GCTATCTTCAAGATCANNNNNN), 200 μM dNTPs, and 1 unit of *Taq* polymerase. Round A consisted of five cycles at 94°C for 1 min, 25°C for 1.5 min, 25–72°C over 7 min, and 72°C for 3 min using *Taq* polymerase (BMB). For round B, the reaction volume was brought up to 100 μl for a final concentration of 2 μM of primer B (GCTATCTTCAAGATCA), 200 μM dNTPs, and 4 units of *Taq* polymerase. Round B consisted of 30 cycles of 94°C for 1 min, 56°C for 2 min, and 72°C for 3 min. The amplification was performed in 96-well plates using crude phage lysates as the templates, resulting in an amplification of both the 35-kb λ vector and the 5-kb to 15-kb yeast insert sequences as a distribution of PCR products between 250 bp and 1500 bp in length.

The PCR products were purified and transferred into TE (10 mM Tris, 1 mM EDTA at pH 8.0) buffer using Sephadex G50 gel filtration (Pharmacia) and evaporated to dryness at room temperature overnight. Each of the 864 amplified λ clones was rehydrated in 15 μl of 3× SSC (20× SSC = 3 M NaCl, 0.3 M Na_3 citrate) in preparation for spotting onto the glass under normal room temperature conditions.

Preparation of DNA Microarrays

The microarrays were fabricated on poly-L-lysine coated microscope slides (Sigma). A custom-built arraying machine, consisting of four tweezer-like printing tips mounted 9 mm apart on a computer-controlled robotic stage (Shalon 1996), loaded 1 μl of the concentrated PCR product directly from corresponding clusters of four wells of 96-well storage plates and deposited ~5 nl of each sample onto each of 40 slides. Surface tension loaded the sample into the printing tip directly from the microwell plate and held the sample in the tip during the printing operation. Printing was achieved by lightly tapping the tip against the glass surface. The open-capillary design allowed for rapid rinsing and drying of the tips between samples. Figure 3 shows the layout of the arraying machine. Figure 4 shows a detailed view of the four printing tips and the staggered printing pattern on the microscope slides. Adjacent samples were spotted 380 μm apart on the slides. After each set of four samples was printed onto 40 slides, the printing tips were rinsed with a jet of water for 2 sec and then dried by lowering the tips onto a sponge for 2 sec. The process was repeated for all 864 samples and eight control spots.

After the spotting operation was complete, the slides were rehydrated in a humid chamber at room temperature for 2 hr, baked in an 80°C vacuum oven for 2 hr, then rinsed in 0.1% sodium dodecyl sulfate (SDS) to remove unadsorbed DNA. To reduce nonspecific adsorption of the labeled hybridization probe to the poly-L-lysine coated glass surface, the slides were treated with succinic anhydride. One gram of succinic anhydride was dissolved in 100 ml of 1-methyl-2-pyrrolidinone and then 100 ml of 0.2 M boric acid (pH 8.0) was added. The arrays were soaked in this solution for 10 min and then rinsed in distilled water four times for 5 min each. Immediately before use, the arrayed DNA elements were denatured by placing the slide in distilled water at 90°C for 2 min.

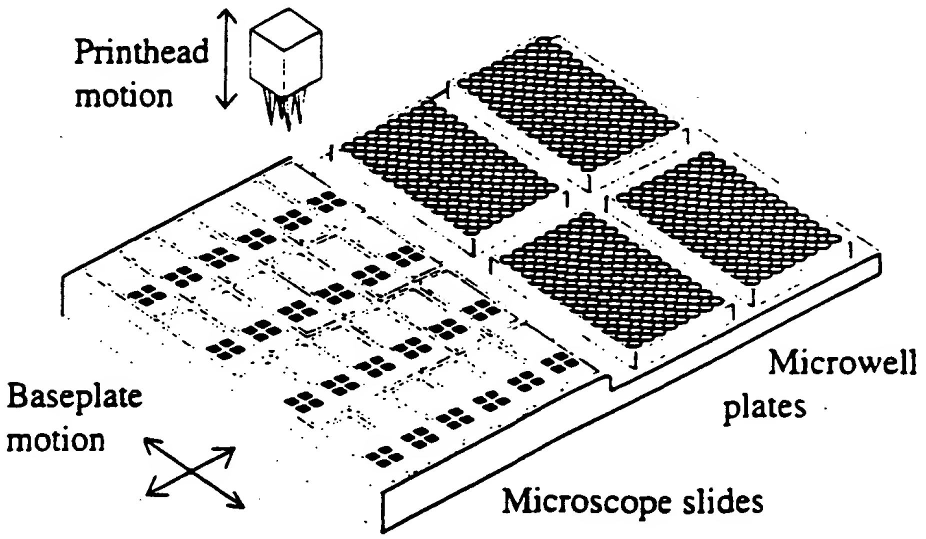

Figure 3 The layout of the arraying machine. All motions are under computer control. For more details of the arraying machine, see web page http://cmgm.stanford.edu/pbrown.

Amplification and Labeling of Hybridization Probe

The 16 chromosomes of *Saccharomyces cerevisiae* were separated using a contour-clamped homogeneous electric field (CHEF) agarose gel apparatus (Bio-Rad) (Chu et al. 1986). The 6 largest chromosomes were isolated in one gel slice and the smallest ten chromosomes in a second gel slice. The DNA from each slice was recovered using a gel extraction kit

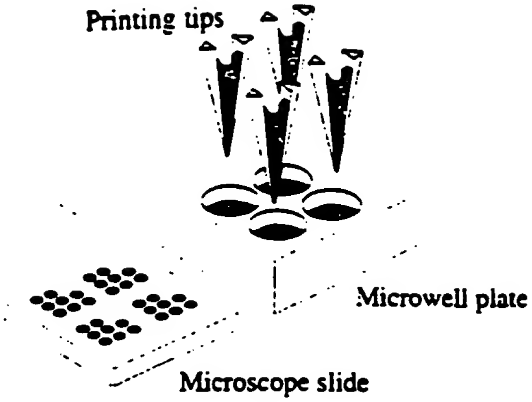

Figure 4 A close-up view of the four open-capillary printing tips. The tips are 9 mm apart and fit into four adjacent wells of a standard microwell plate and print arrays in a staggered fashion on microscope slides. For more details of the printing tips, see web page http://cmgm.stanford.edu/pbrown.

(Qiagen) and randomly amplified in a manner similar to that used in amplifying the target λ clones (Grothues et al. 1993). The main difference between this amplification procedure and the one used for the λ array elements is a filtration step between rounds A and B to remove primer-dimers and the use of a random 9-mer 3′ end on primer A. Following amplification, 2.5 μg of each of the amplified chromosome pools were separately random-primer labeled using Klenow polymerase (Amersham) with a lissamine-conjugated nucleotide analog (DuPont NEN) for the pool containing the 6 largest chromosomes and with a fluorescein-conjugated nucleotide analog (BMB) for the pool containing the smallest 10 chromosomes. The two fluorescent-labeled pools were mixed and concentrated using an ultrafiltration device (Amicon).

Hybridization

Five micrograms of the hybridization probe, consisting of both chromosome pools in 7.5 μl of TE, was denatured in a boiling water bath and then snap-cooled on ice. Concentrated hybridization solution (2.5 μl) was added to a final concentration of 5× SSC/0.1% SDS. The entire 10 μl of probe solution was transferred to the array surface, covered with a coverslip, placed in a custom-built single-slide humidity chamber, and incubated in a 60°C water bath for 12 hr. The custom-built waterproof slide chamber has a cavity just slightly bigger than a microscope slide and was kept at 100% humidity internally by the addition of 2 μl of water in a corner of the chamber. The slide was rinsed in 5× SSC/0.1% SDS for 5 min and then in 0.2× SSC/0.1% SDS for 5 min. All rinses were at room temperature. The array was then air dried, and a drop of antifade (Molecular Probes) was applied to the array under a 24-mm × 30-mm coverslip in preparation for scanning.

Detection and Analysis

A custom-built laser scanner was used to detect the two-color fluorescence hybridization signals from 1.8-cm × 1.8-cm arrays at 20-μm resolution. The glass substrate slide was mounted on a computer-controlled, two-axis translation stage (PM-500, Newport, Irvine, CA) that scanned the array over an upward-facing microscope objective (20×, 0.75NA Fluor, Nikon, Melville, NY) in a bi-directional raster pattern. A water-cooled Argon/Krypton laser (Innova 70 Spectrum, Coherent, Palo Alto, CA), operated in multiline mode, allowed for simultaneous specimen illumination at 488.0 nm and 568.2 nm. These two lines were isolated by a 488/568 dual-band excitation filter (Chroma Technology, Brattleboro, VT). An epifluorescence configuration with a dual-band 488/568 primary beam splitter (Chroma) excited both fluorophores simultaneously and directed fluorescence emissions toward the two-channel detector. Emissions were split by a secondary dichroic mirror with a 565 transition wavelength onto two multialkali cathode photomultiplier tubes (PMT; R928, Hamamatsu, Bridgewater, NJ), one with an HQ535/50 bandpass barrier filter and the other with a D630/60 bandpass barrier filter (Chroma). Preamplified PMT signals were read into a personal computer using a 12-bit analog-to-digital conversion board (RTI-834, Analog Devices, Norwood, MA), displayed in a graphics window, and stored to disk for further rendering and analysis. The back aperture of the 20× objective was deliberately underfilled by the illuminating laser beam to produce a large-diameter illuminating spot at the specimen (5-μm to 10-μm half-width). Stage scanning velocity was 100 mm/sec, and PMT signals were digitized at 100 μsec intervals. Two successive readings were summed for each pixel, such that pixel spacing in the final image was 20 μm. Beam power at the specimen was -5 mW for each of the two lines.

The scanned image was despeckled using a graphics program (Hijaak Graphics Suite) and then analyzed using a custom image gridding program that created a spreadsheet of the average red and green hybridization intensities for each spot. The red and green hybridization intensities were corrected for optical cross talk between the fluorescein and lissamine channels, using experimentally determined coefficients.

ACKNOWLEDGMENTS

This research was supported by grant HG00450 from the National Institutes of Health–National Center for Human Genome Research, a National Science Foundation graduate fellowship to D.S., and by the Howard Hughes Medical Institute. P.O.B. is an assistant investigator of the Howard Hughes Medical Institute. We thank John Mulligan and John McCusker for help in preparing and amplifying the λ clones used in the arrays, Ren Xin Xia for writing the scanner control software and the image gridding and automatic karyotyping programs, Jeff van Ness at Darwin Molecular Corporation for suggesting the use of succinic anhydride, Stan Nelson, Linda McAllister, Joe deRisi, and Lolita Penland for helpful suggestions in the course of this work, and Joe deRisi and Linda McAllister for helpful comments on the manuscript.

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Received March 4, 1996; accepted in revised form May 9, 1996.